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Alterations in Levels of DnaK and GroEL Result in Diminished Survival and Adherence of Stressed *Haemophilus ducreyi*

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***Haemophilus ducreyi* is a hemin-requiring bacterium causing the genital ulcer disease chancroid. Previously we demonstrated that the heat shock protein GroEL was immunogenic and possibly highly expressed in a mammalian host. The present study was initiated to (i) determine the relative amounts of GroEL expressed by *H. ducreyi* during in vitro exposure to stresses and (ii) evaluate whether a high level of GroEL is directly or indirectly required for survival and adherence of stressed *H. ducreyi*. Using scanning densitometry of sodium dodecyl sulfate-polyacrylamide gel electrophoresis protein profiles, we found that *H. ducreyi* expressed high basal levels of GroEL, averaging fivefold greater than in *Escherichia coli*. These high GroEL levels increased up to twofold upon exposure of the organism to heat shock or high levels of hydrogen peroxide and during adherence to two human genital cell lines. Furthermore, when the gene for DnaK was present on a multicopy plasmid in *H. ducreyi*, a 1.8-fold increase in DnaK and a 2.3-fold reduction in GroEL were seen. These results suggest that DnaK serves as a negative modulator of *H. ducreyi* GroEL. Subsequently we found that *H. ducreyi* with lower GroEL had diminished ability to survive when challenged by heat and oxidative stresses. In addition, the long, parallel chains characteristic of virulent strains of *H. ducreyi* were absent when GroEL was lowered, so that fewer bacterial cells adhered to the human cells. These results suggest that the unusually high basal levels of GroEL are involved, either directly or indirectly, in the survival, chaining, and adherence of *H. ducreyi* in the presence of the combined stresses of the host environment.**

The sexually transmitted pathogen *Haemophilus ducreyi* grows best in vitro at 32 to 33°C, with viability decreasing rapidly at 37°C. Viable *H. ducreyi* is not able to spread systemically from skin lesions during human infection, suggesting that the temperature sensitivity seen in vitro contributes to the organism's inability to persist at 37°C in vivo. Preferential survival of *H. ducreyi* on the (lower-temperature) surface of the host may enhance its transmission during sexual intercourse. However, during in vivo growth when transient exposures to the core temperature of human hosts would occur, it is likely that heat shock proteins play a critical role in survival of *H. ducreyi.*

Owing to their involvement in protein folding and renaturation, the molecular chaperones and chaperonins that are heat shock proteins stabilize essential and virulence-related proteins in bacterial pathogens during exposure to environmental stress (14, 15). A general role for the Hsp60 (GroEL and GroES) and Hsp70 (DnaK and DnaJ) families in the folding-assembly pathways for many proteins has been established in *Escherichia coli* (7, 14, 37, 39). Specifically, proteins from the Hsp70 and Hsp60 families are involved in the synthesis of flagellin in *E. coli* (32) and *Borrelia burgdorferi* (31). In addition, GroES and GroEL (Hsp60) are involved in efficient folding of gene regulators in *Klebsiella pneumoniae* (16), *Vibrio fischeri* (7), and *Rhizobium meliloti* (13). And in *Helicobacter pylori*, GroEL may be involved in extracellular stabilization of the virulence-related enzyme urease (8). These results suggest that the role of heat shock proteins in facilitating protein secondary and tertiary structure is critically important for bacterial pathogens in their survival and expression of virulence-related proteins.

The prokaryotic heat shock response, which involves transient expression of Hsp60, Hsp70, and other heat shock proteins, is subject to both positive and negative regulation. A consensus sequence for *E. coli* heat shock gene promoters was described in 1985 (5), suggesting that a specific sigma factor was involved as a positive regulator. Moreover, bacteria that overproduced DnaK (Hsp70) protein at all temperatures had a drastically reduced heat shock response at high temperature (34), suggesting that DnaK was an inhibitor of the heat shock response in *E. coli*. Subsequently, the involvement of DnaK, DnaJ, and GrpE in the negative modulation of the *E. coli* heat shock response through interaction with the positive regulator sigma-32 has been well documented (2, 23; reviewed in references 15 and 38).

A previous report from our laboratory described the *H. ducreyi groE* heat shock operon, encoding GroES and GroEL (28). RNA and protein analyses demonstrated that this operon is highly expressed in both exponential- and stationary-phase *H. ducreyi* (3, 28), and GroEL is immunogenic in human chancroid patients and in animals inoculated with live *H. ducreyi* (3, 28, 36). The *H. ducreyi groE* transcriptional start site is preceded by a promoter with significant homology to the *E. coli* consensus heat shock promoter recognized by sigma-32 (5). This homology suggested that DnaK is involved as a negative modulator of heat shock gene expression via interaction with the sigma-32 homolog.

In the present study, we found a basal level of *H. ducreyi* GroEL that was up to fivefold greater than the GroEL basal level found in *E. coli*. We also demonstrated that GroEL levels were further increased during exposure of *H. ducreyi* to environmental stresses such as heat shock. In addition, when DnaK was overexpressed in *H. ducreyi*, GroEL expression decreased.

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H. ducreyi with lowered GroEL was then used to evaluate whether decreases in GroEL would influence survival of stressed and unstressed *H. ducreyi.*

MATERIALS AND METHODS

Bacteria and media. The virulent *H. ducreyi* strain 35000 (ATCC 33922) and the avirulent strains A76 and A77 were stored frozen at −70°C in 15% glycerol broth. Solid media for the growth of *H. ducreyi* included (i) CA-RB blood agar, prepared with Casman agar base, 5% rabbit blood, and 10% fetal bovine serum (FBS), and (ii) a clear agar containing catalase as the source of hemin (35). Medium bases and other chemical components were purchased from Difco Laboratories, Detroit, Mich., or Sigma Chemical Co., St. Louis, Mo.

A previously described hemin-rich broth (HRB-1) (1) containing 200 μg of hemin per ml and 10% FBS was used in the early part of this study. As work progressed, midhemin broth, containing brain heart infusion broth base, 1% IsoVitaleX, 5% FBS, and 25 μg of hemin per ml, was used.

Determination of bacterial growth and viability. Stocks of *H. ducreyi* stored at −70°C in midhemin broth with 15% glycerol were used to inoculate other tubes of broth. Because clumping of *H. ducreyi* previously grown on solid media interferes with growth density determinations, avoidance of solid media by transfer of growth from broth to broth resulted in fairly homogeneous bacterial suspensions that were satisfactory for determining the turbidity of bacterial growth by reading the absorbance at 540 nm. The number of viable CFU was estimated by performing serial dilutions on duplicate samples and plating aliquots on CA-RB or catalase agar plates.

SDS-PAGE. The protein concentrations of sonicated bacterial suspensions were determined with protein assay reagent (Bio-Rad Laboratories, Richmond, Calif.). The protein preparations were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using standard methods (30). Gel bands were analyzed by scanning densitometry (RFLP Scan; Scanalytics, Billerica, Mass.). To control for variations in loading of the protein samples, values for the bands of interest were determined as a percentage of the total protein bands for each lane.

Metabolic labeling of *H. ducreyi* proteins with [^{35}S]methionine. *H. ducreyi* previously grown for 18 to 24 h at 32°C in broth was pelleted by centrifugation in a microcentrifuge and resuspended in Eagle's minimal essential medium with 5% fetal bovine serum (E-FBS) without methionine (E-FBS^{-met}) containing 10 μCi of L-[^{35}S]methionine (Amersham Corp.). Following a 30- to 45-min labeling period under the conditions described in each figure legend, the samples were placed on ice and washed three times in cold phosphate-buffered saline (PBS), and the cells were resuspended in 1× SDS protein stop mix (19). The labeled proteins were separated on SDS-PAGE gels. Labeled protein bands were quantitated by autoradiography and scanning densitometry or by phosphorimaging (PhosphorImager and ImageQuant; Molecular Dynamics, Sunnyvale, Calif.). To control for variations in loading and exposure, values for the bands of interest were determined as a percentage of the total protein bands for each lane. The percentages from two to three experiments were averaged and compared for determining ratios of increase.

Subcloning *H. ducreyi dnaK* into pLS88 and electroporation of pHDk4 into *H. ducreyi.* A 5.4-kb *Eco*RI fragment containing *H. ducreyi dnaK* (unpublished data) (GenBank/EMBL accession number U25996) was cloned into the *H. ducreyi* shuttle plasmid pLS88 (6), which is present in approximately 25 to 50 copies per cell. Following ligation and transformation into *E. coli* JM109, pHDk4 and the control plasmid, pLS88, were purified with a Plasmid Midi Kit (Qiagen, Inc., Chatsworth, Calif.) and used for electroporation into *H. ducreyi* 35000 as previously described (17). In brief, the cells were electroporated with a Bio-Rad Gene Pulser and 0.1-cm cuvettes at 2.5 kV, 25 μF, and 400 Ω with 1 μg of plasmid DNA and then plated on catalase agar plates for a 4.5-h recovery period at 32°C. The growth was harvested from the nonselective plates and replated on selective catalase agar plates with 40 μg of streptomycin per ml. Transformants were isolated following 2 to 3 days of incubation at 32°C. *H. ducreyi* transformants containing pHDk4 were designated 35000-KJ, and those containing pLS88 were designated 35000-PL. In pHDk4, the DnaK gene was preceded by its own promoter(s) and thus was subject to normal cellular control.

Challenge of *H. ducreyi* with hydrogen peroxide. Suspensions of approximately 10^6 CFU of *H. ducreyi* suspended in 20 μl of midhemin broth were added to 40 μl of hydrogen peroxide so that the final concentration varied from 0.15 to 2.5 mM. Following a 30-min challenge at 32 or 37°C, 2 ml of midhemin broth was added to each well to dilute the hydrogen peroxide concentrations. The plates were then incubated for 48 h at the challenge temperature. Bacterial growth was determined by assessing the visual turbidity.

Adherence to human cells. An in vitro cell culture adherence assay that correlated with the organism's ability to cause lesions in the rabbit model was developed in this laboratory (27). In brief, tissue culture cells were grown for 2 to 3 days on 16-mm coverslips in 24-well plates in E-FBS. The ratio of infecting bacteria to eukaryotic cells was approximately 4:1. Following an adherence period of 2 to 4 h at 32°C in 5% CO_2, the coverslips were washed three times with E-FBS to eliminate nonadherent bacteria, and the plates were reincubated at 32°C for a total of 24 h. The coverslips were then washed three times with PBS, fixed with methanol, stained with Diff-Quik stain (American Scientific Products, McGaw Park, Ill.), and permanently mounted onto slides. Interactions between *H. ducreyi* and the tissue culture cells were evaluated by light microscopy with a Labphot microscope (Nikon Corp., Tokyo, Japan). Photographs were taken at a magnification of ×1,000 with Kodak ASA 100 and 125 high-contrast color print film.

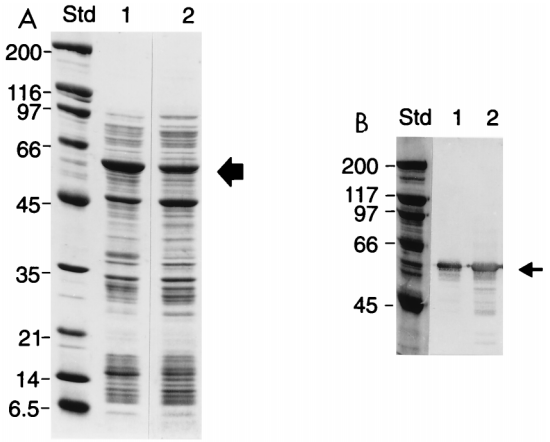

FIG. 1. Protein profiles of *H. ducreyi*. (A) Coomassie blue-stained gel. Fifteen micrograms of protein from sonicated whole *H. ducreyi* was subjected to SDS-PAGE on an 8 to 18% gradient gel, and the gel was stained with Coomassie blue. Lanes 1 and 2 contain protein from *H. ducreyi* 35000 and A76, respectively, grown on CA-RB slants for 24 h at 32°C. (B) Reactivity of *H. ducreyi* GroEL with antiserum to *E. coli* GroEL. Five micrograms of total cellular protein from *E. coli* NM522 (lane 1) and *H. ducreyi* 35000 (lane 2), previously grown on CA-RB slants, was subjected to SDS–7.5% PAGE and then immunoblotted with polyclonal antiserum to *E. coli* GroEL (immunoglobulin G) (Epicentre Technologies, Madison, Wis.). *E. coli* was incubated at 37°C, while *H. ducreyi* was incubated at 33°C. In both panels, the sizes of the protein standards (Std) are indicated (in kilodaltons). Arrows indicate the prominent 60-kDa protein.

Expression of GroEL in adherent *H. ducreyi*. Two strains of *H. ducreyi* were grown in midhemin broth for 18 h at 32°C prior to inoculation of monolayers of Caski cells or human foreskin fibroblasts (HFF) in E-FBS. After 4 h of adherence at 32°C, nonadherent bacteria were washed off, and the incubation was continued for another 20 h at the same temperature. At 24 h, fresh E-FBS with 50 μg of cycloheximide per ml was added and incubated for 45 min (to eliminate eukaryotic protein synthesis), followed by removal of this medium and addition of E-FBS^{-met} containing 50 μg of cycloheximide per ml and 20 μCi of [^{35}S]methionine per ml. The incubation was then continued for another 45 min at 32°C, and washed adherent cells were solubilized in 1× SDS protein stop mix. As described above, the labeled proteins were separated on SDS-PAGE gels, and labeled bands were quantitated by autoradiography and scanning densitometry.

RESULTS

A GroEL homolog is highly expressed in *H. ducreyi*. The most predominant protein present in *H. ducreyi* strains grown at 32°C (Fig. 1A) has a molecular mass of approximately 60 kDa. Scanning densitometry shows that this predominant protein represents an average of 10% of the total soluble protein in the virulent strain 35000 (lane 1) and an average of 6% in the avirulent strains A76 (lane 2) and A77 (data not shown). This protein was identified as the GroEL (Hsp60) homolog by reactivity in Western blots with antiserum to *E. coli* GroEL (Fig. 1B) and will be referred to in this report as *H. ducreyi* GroEL.

GroEL levels increase when *H. ducreyi* is exposed to common environmental stresses. (i) Heat shock. [^{35}S]methionine labeling of *H. ducreyi* proteins indicated that the level of GroEL increased after heat shock in *H. ducreyi* an average of 1.9-fold at 37°C, 2.1-fold at 40°C, and 2.3-fold at 42°C. A representative experiment using strains 35000 and A77 is shown in Fig. 2.

Two other proteins also shown in Fig. 2 were induced by heat shock: a 78-kDa protein, identified as the *H. ducreyi* DnaK

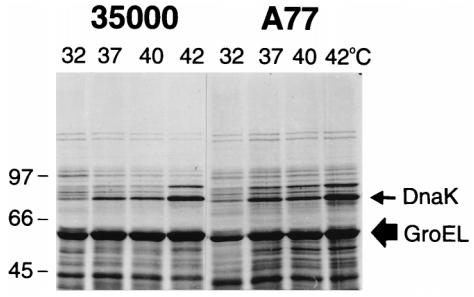

FIG. 2. Expression of heat shock proteins in *H. ducreyi*. Two strains of *H. ducreyi* were grown in HRB-1 for 18 h at 32°C, washed and resuspended in E-FBS^{-Met}, and then heat shocked for 45 min in the presence of [^{35}S]methionine at 37, 40, and 42°C. Equal numbers of counts (5×10^4 cpm) were loaded per lane for SDS–7.5% PAGE. The autoradiograph was exposed for 4 days. The sizes of the protein standards are indicated (in kilodaltons).

homolog by reactivity in Western blots with antiserum to *E. coli* DnaK (data not shown), and an unidentified 87-kDa protein, similar in size to a 90-kDa protease induced by heat shock in *E. coli* (15).

(ii) Stationary phase. The amount of GroEL present in *H. ducreyi* at various times during the growth curve was determined with SDS-PAGE gels (Fig. 3). There was a gradual increase in the level of GroEL, as determined by scanning densitometry (2.2-fold in strain 35000 and 1.4-fold in A77) as the cells entered stationary phase (36 to 44 h [28]).

(iii) Oxidative stress: exposure to hydrogen peroxide. GroEL expression was evaluated following a 45-min exposure of the catalase-negative *H. ducreyi* to low (0.2 mM) and high (2 mM) concentrations of hydrogen peroxide by using [^{35}S]methionine labeling of proteins. GroEL expression was found to increase slightly in strain 35000 following exposure to both concentrations of hydrogen peroxide (1.2- and 1.4-fold increases, respectively) (data not shown). Similar increases were seen with strain A77.

(iv) Adherent *H. ducreyi*. An in vitro assay that differentiated virulent and avirulent strains of *H. ducreyi* by their adherence phenotype at 32°C was previously developed in this laboratory (27). In this assay, both virulent strain 35000 and avirulent strain A77 were capable of adhering to the human genital cells. However, substantial differences in the grouping of the adherent bacteria were noted. Strain 35000 adhered as long, thick ropes of parallel chains of bacilli (Fig. 4A). These thick mats of bacteria were seen intertwined around and between the human cells and appeared similar to the "railroad tracks" or "shoals of fish" characteristic of *H. ducreyi* seen in smears taken from chancroidal ulcers and buboes (36). In contrast, the long, parallel chains were not seen with avirulent strain A77. Instead, these bacteria adhered to the human cells in small groups and short chains (Fig. 4B), even after 24 h of interaction.

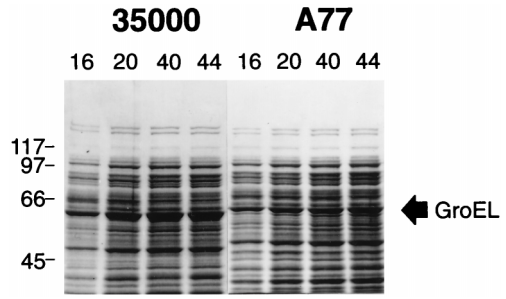

FIG. 3. Expression of *H. ducreyi* GroEL during stationary phase: Coomassie blue-stained SDS–7.5% PAGE gels of sonicated whole bacteria obtained from two strains of *H. ducreyi* previously grown on CA-RB slants at 32°C for 16, 20, 40, and 44 h. Each lane contained 15 μg of protein. The sizes of the protein standards are indicated (in kilodaltons).

This in vitro cell culture assay was used in the present study to determine whether expression of GroEL in *H. ducreyi* was influenced by adherence to human cells. As compared with the percentage of [^{35}S]methionine-labeled GroEL in *H. ducreyi* suspended in E-FBS, the percentage of GroEL in adherent cells of strain 35000 increased 1.6- and 1.9-fold during interaction with Caski cells and HFF cells, respectively (data not shown). GroEL expression also increased in adherent cells of strain A77, with GroEL percentages increasing 1.4- and 2.5-fold during interaction with Caski cells and HFF cells, respectively.

Presumptive involvement of *H. ducreyi* DnaK in negative modulation of expression of the GroE proteins. In *E. coli*, GroEL expression is modulated by proper functioning and levels of DnaK (23). *H. ducreyi* DnaK was identified by us and others as a 78-kDa heat-inducible protein (3) (Fig. 2). The gene for *H. ducreyi* DnaK was amplified by PCR and a set of degenerate oligonucleotide primers for highly conserved regions in Hsp70 homologs (unpublished data). Subsequently, a chromosomal fragment containing *H. ducreyi dnaK* was subcloned into a multicopy *H. ducreyi* plasmid, pLS88, to create pHDk4, and both plasmids were transformed into *H. ducreyi* 35000 by electroporation, resulting in 35000-KJ, containing pHDk4, and 35000-PL, containing pLS88.

As shown in Fig. 5A, an increase in expression of DnaK in *H. ducreyi* 35000-KJ correlated with decreased expression of GroEL. In addition to GroEL, decreased expression of both GroES and the unidentified 87-kDa heat-induced protein (Fig. 2) was observed in strain 35000-KJ. Analysis of gels by phosphorimaging showed that basal levels of DnaK in *H. ducreyi* 35000-KJ increased 1.8-fold over that seen in 35000-PL, while basal levels of GroEL decreased 2.3-fold (Fig. 5B). Such decreases in expression of heat shock proteins in *H. ducreyi* following overexpression of DnaK provide presumptive evidence that, as in *E. coli*, DnaK functions as a negative modulator of the heat shock response.

Alterations in heat shock protein expression results in diminished survival and adherence of stressed *H. ducreyi*. (i) Bacterial growth. Growth at different temperatures was compared for *H. ducreyi* 35000-KJ and 35000-PL. When absorbance readings were compared at 24, 48, 72, and 96 h, there were no differences in cell densities of the two strains incubated at 32°C. However, at 37°C, the strain with lowered GroEL (35000-KJ) grew in midhemin broth to cell densities that averaged only 42% of cell densities for strain 35000-PL, which expressed GroEL levels normal for *H. ducreyi.*

(ii) Cell viability. Cell viability (CFU) was also compared for strains 35000-KJ and 35000-PL. Duplicate tubes of midhemin broth were inoculated with an 18-h 32°C broth culture of each strain and incubated at either 32 or 37°C. Following 24-h incubation, the viable counts for strains 35000-KJ and 35000-PL incubated at 37°C were 3 and 41%, respectively, of those in the identical suspensions grown at 32°C. Differences in survival were also seen when 18-h 32°C broth cultures were heat shocked for 3 h at 40°C; only 2% of the original inoculum of strain 35000-KJ survived, compared with 26% survival for 35000-PL. Additionally, after 24 h of heat shock at 37°C, viable counts for 35000-KJ were 0.04% of the original inoculum, compared with 18% for 35000-PL. These results clearly show that growth and/or survival of *H. ducreyi* at 37°C is adversely affected by the presence of the plasmid containing the gene for

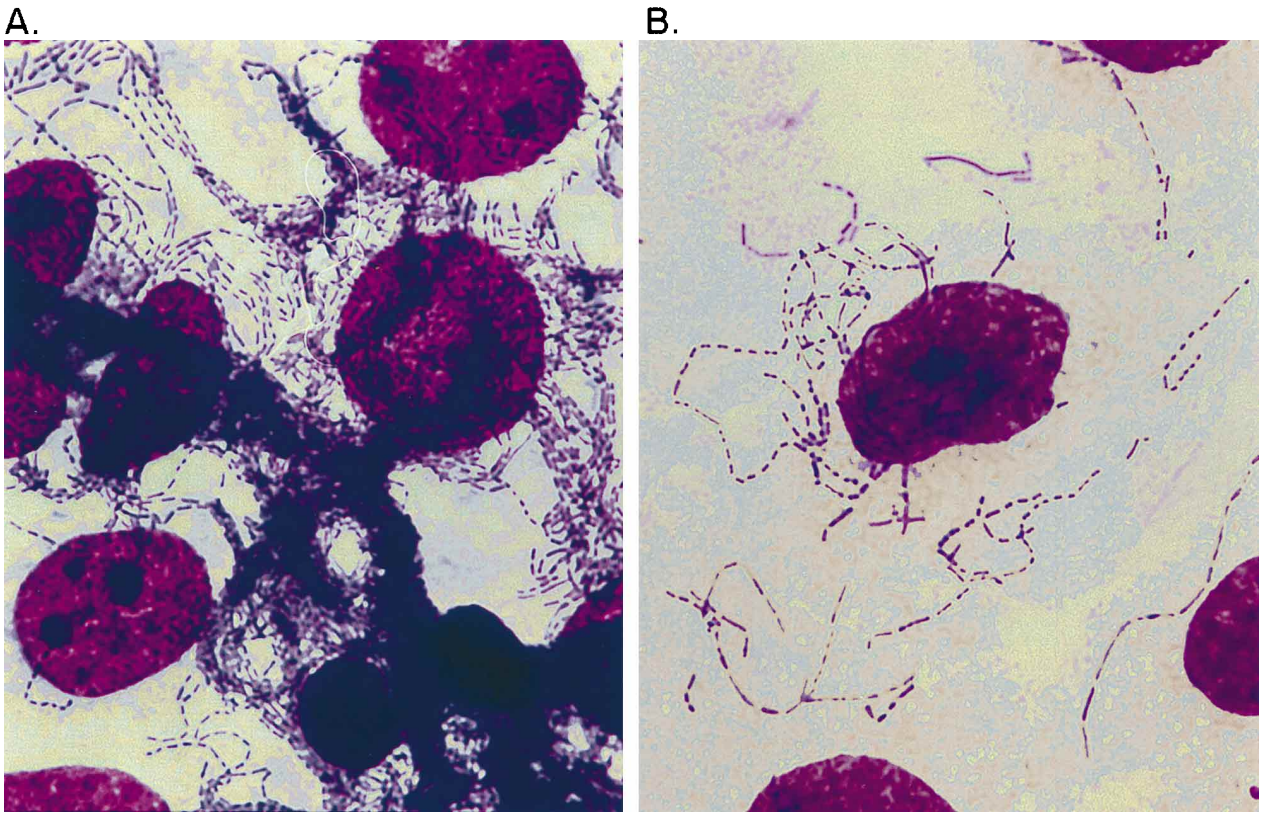

FIG. 4. Adherence of *H. ducreyi* to Caski cervical carcinoma cells. *H. ducreyi* 35000 (A) and A77 (B), previously grown for 24 h in HRB-1, were used to inoculate monolayers of Caski cells (approximately 4 bacteria/Caski cell). Following 1.5 h of adherence at 33°C in E-FBS, nonadherent bacteria were washed off, fresh E-FBS was added, and incubation was continued for 22.5 h at 33°C before the coverslips were washed and stained with Diff-Quik. Adherent cells were photographed with a Nikon Labphot microscope under oil immersion (original magnification, ×1,000).

DnaK, and the adverse affect may be due to the decrease in GroEL.

(iii) Bacterial growth following hydrogen peroxide challenge. Visible bacterial growth in plastic 24-well tissue culture wells containing twofold dilutions of hydrogen peroxide (2.5 to 0.15 mM) was used to compare the sensitivity of the two strains of *H. ducreyi* to oxidative stress. As shown in Table 1, the cells with low GroEL (35000-KJ) were two- to fourfold more sensitive to oxidative stress than were cells with normal GroEL levels (35000-PL) when challenge occurred at 37°C. Interestingly, stationary-phase *H. ducreyi*, regardless of GroEL level, were more sensitive than log-phase cells to combined heat and oxidative challenges. In contrast, stationary-phase *E. coli* are more resistant to oxidative stress than vegetative-phase cells (11).

(iv) Adherence to cultured human cells. The tissue culture system that was described previously (Fig. 4) was used to determine whether *H. ducreyi* 35000-KJ would be capable of adherence to HFF. As shown in Fig. 6B, *H. ducreyi* 35000-KJ was not present on the HFF monolayer in quantities comparable to that of strain 35000-PL (Fig. 6A). Differences in cellular morphology of the adherent *H. ducreyi* 35000-KJ and 35000-PL can also be seen in Fig. 6. The distinctive chaining characteristic of virulent strains of *H. ducreyi* is also diminished in 35000-KJ. These results suggest either direct or indirect involvement of GroEL with bacterial proteins participating in chaining and adherence.

DISCUSSION

The highly conserved 60-kDa molecular chaperonin GroEL was found to be the predominant protein in *H. ducreyi*, representing approximately 10% of total cellular proteins at 32°C, with an increase to 20% following 37 to 40°C heat shock. This high level of GroEL is significantly greater than in other bacteria. In *E. coli*, GroEL represents 1.6 to 2% of the total cellular protein at 37°C, with an increase to 12 to 15% following 42°C heat shock (18, 26). Similar low basal levels of GroEL (approximately 2% of total cellular proteins in stained protein gels, confirmed by Western blots) were noted by us in seven strains of *Haemophilus influenzae* and *Haemophilus parainfluenzae* (data not shown). Thus, the high basal levels of GroEL in *H. ducreyi* are not shared by the other *Haemophilus* species or *E. coli*, suggesting that this protein plays a more prominent role in the physiology of *H. ducreyi* than in these other bacterial species.

Some heat shock proteins, including the GroE homologs, can be induced by the nutrient starvation associated with the onset of stationary phase (20, 33). In addition, low-level induction of the *groE* operon by oxidative stress has been described in other gram-negative bacteria (9, 11, 22), and GroEL expression has been shown to be upregulated in bacterial pathogens during growth in mammalian cells (4, 10). In the present study, we found that GroEL was induced in *H. ducreyi* by heat shock and also during exposure to the following conditions: stationary-phase growth, exposure to hydrogen peroxide, and adherence to human cells.

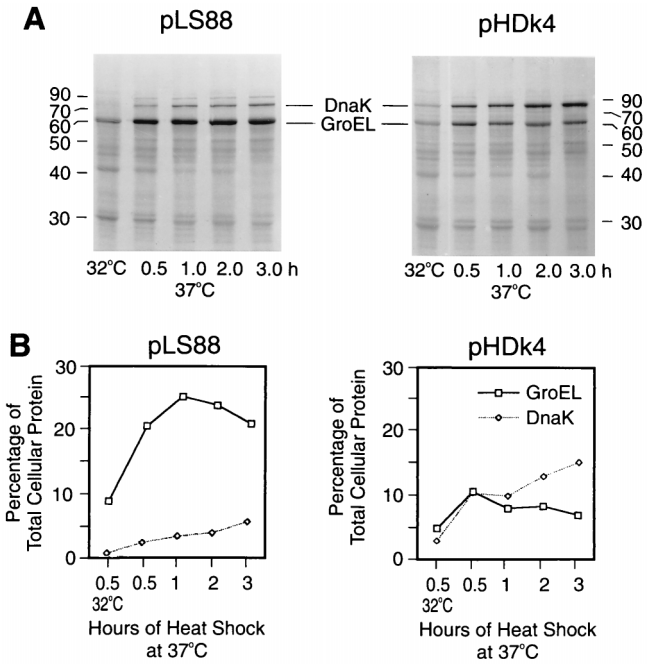

FIG. 5. Effect of increased DnaK on heat shock protein expression in *H. ducreyi*. (A) Protein gels. *H. ducreyi* 35000-PL (carrying pLS88) and 35000-KJ (carrying pHDk4) were grown in Mueller-Hinton broth for 18 h at 32°C and then heat shocked for 0.5, 1, 2, and 3 h at 37°C. The bacteria were resuspended in E-FBS^{-Met} with [^{35}S]methionine for the last 30 min of each heat shock period. The two 8 to 18% gradient gels were loaded with 10^4 dpm per lane. The autoradiographs were exposed for 1 day. The sizes of the protein standards are indicated (in kilodaltons). (B) GroEL and DnaK as percentages of total cellular protein. Protein samples, obtained as described for panel A, were separated on 8 to 18% gradient gels, and labeled bands were quantitated by phosphorimaging. The values for the GroEL and DnaK bands were determined as a percentage of the total protein bands in each lane. The values shown are the averages from two experiments.

TABLE 1. Visible bacterial growth of *H. ducreyi* 35000-PL (PL) and 35000-KJ (KJ) following combined heat and hydrogen peroxide challenge

Phase, temp, and *H. ducreyi* strain	Growth[a] with indicated H_2O_2 concn (mM)					
	2.5	1.25	0.6	0.3	0.15	0 (PBS)
Log phase						
32°C						
PL	−	+++	+++	+++	+++	+++
KJ	−	+++	+++	+++	+++	+++
37°C						
PL	−	−	+	+++	+++	+++
KJ	−	−	−	+	+	+++
Stationary phase						
32°C						
PL	−	+	+++	+++	ND	+++
KJ	−	+	+++	+++	ND	+++
37°C						
PL	−	−	−	+	ND	+++
KJ	−	−	−	−	ND	+

[a] Interpretation of bacterial growth in a 24-well plate after 48 h of incubation: +++, dense visible turbidity; +, light visible turbidity; −, no visible turbidity; ND, not done.

The promoter region upstream from the *H. ducreyi groE* transcriptional start site (28) is homologous with consensus heat shock promoters recognized by sigma-32. This homology suggests that sigma-32, the positive regulator of the heat shock response in *E. coli*, is present in *H. ducreyi*. Consensus sigma-32 promoters have been found preceding *groES/EL* and *dnaK* heat shock genes in most gram-negative eubacteria. DnaK, together with DnaJ, is involved in negative modulation of the heat shock response in *E. coli* by binding and inactivating sigma-32 (23). The conserved heat shock promoter seen in the *H. ducreyi groE* operon, suggestive of the presence of a sigma-32 homolog, further indicates that DnaK could be involved in modulation of the *H. ducreyi* heat shock response.

DnaK had previously been found to be present and heat inducible in *H. ducreyi* (3). However, basal levels of GroEL in *H. ducreyi* were up to fivefold greater than basal levels of GroEL in *E. coli*, suggesting that DnaK might not be capable of negative regulation of the heat shock response in *H. ducreyi*. Thus, we cloned and sequenced *H. ducreyi dnaK* (unpublished data) and found that the deduced amino acid sequence of *H. ducreyi* DnaK shared 83% identity and 89% similarity with the *E. coli* homolog. However, we did obtain evidence for preferential transcription and translation of the *H. ducreyi groE* operon compared with the gene for DnaK (unpublished data).

We found that the genes for both DnaK and DnaJ were on the cloned 5.4-kb *H. ducreyi* fragment in pHDk4. However, we were unable to visualize a 40-kDa heat-inducible protein (DnaJ) in *H. ducreyi*. In *E. coli*, the relative level of expression of DnaJ (Hsp40) is much less than that of DnaK, even though the genes are thought to be cotranscribed (2). This disparity has been attributed to infrequent translation initiation of *dnaJ*. Nevertheless, *E. coli* DnaJ has been shown to contribute to negative modulation of the heat shock response, but because of the complex interaction of the molecular chaperones, it has been difficult to tease apart the functions of the individual proteins. Thus, it is not known whether the multiple copies of *dnaJ* present on the cloned fragment contributed to the present results seen in *H. ducreyi.*

However, we did obtain evidence for overexpression of DnaK in *H. ducreyi* 35000-KJ (Fig. 5A), and DnaK overexpression was found to correlate with lowered expression of GroEL and other heat shock proteins. This offers presumptive evidence that DnaK, and perhaps DnaJ, is capable of negatively modulating the heat shock response in *H. ducreyi*. These results are similar to those seen in *E. coli*, when a twofold increase in expression of DnaK and DnaJ led to decreased expression of GroEL (25).

It had previously been shown that GroEL and GroES are essential for growth in *E. coli* (12), and strains lacking sigma-32 cannot grow at temperatures above 20°C, primarily because of the insufficient levels of GroEL and GroES (40). When GroEL was reduced to 25% of that of the wild type in *E. coli*, the growth rate was 20% lower than that of wild-type *E. coli* at 30 and 37°C, with no growth seen at 42°C (21). In another study, a dramatic increase (13.6-fold) in DnaK and DnaJ resulted in reduced cell viability in *E. coli* (2); however, it was not determined whether the adverse effects observed in *E. coli* were due to the high levels of DnaK or to lower levels of GroEL.

In the present study, overexpression of DnaK, and presumably DnaJ, enabled us to analyze the ability of *H. ducreyi* with lowered GroEL to survive in the presence of common environmental stresses. When GroEL levels were lowered in *H. ducreyi*, survival of unstressed organisms was similar to that of wild-type cells. However, when the bacterial cells were stressed by heat, those with low GroEL showed a diminished ability to multiply and also an increased susceptibility to hydrogen peroxide. These results suggest that the high basal level of GroEL seen in wild-type cells is required for *H. ducreyi* to renature

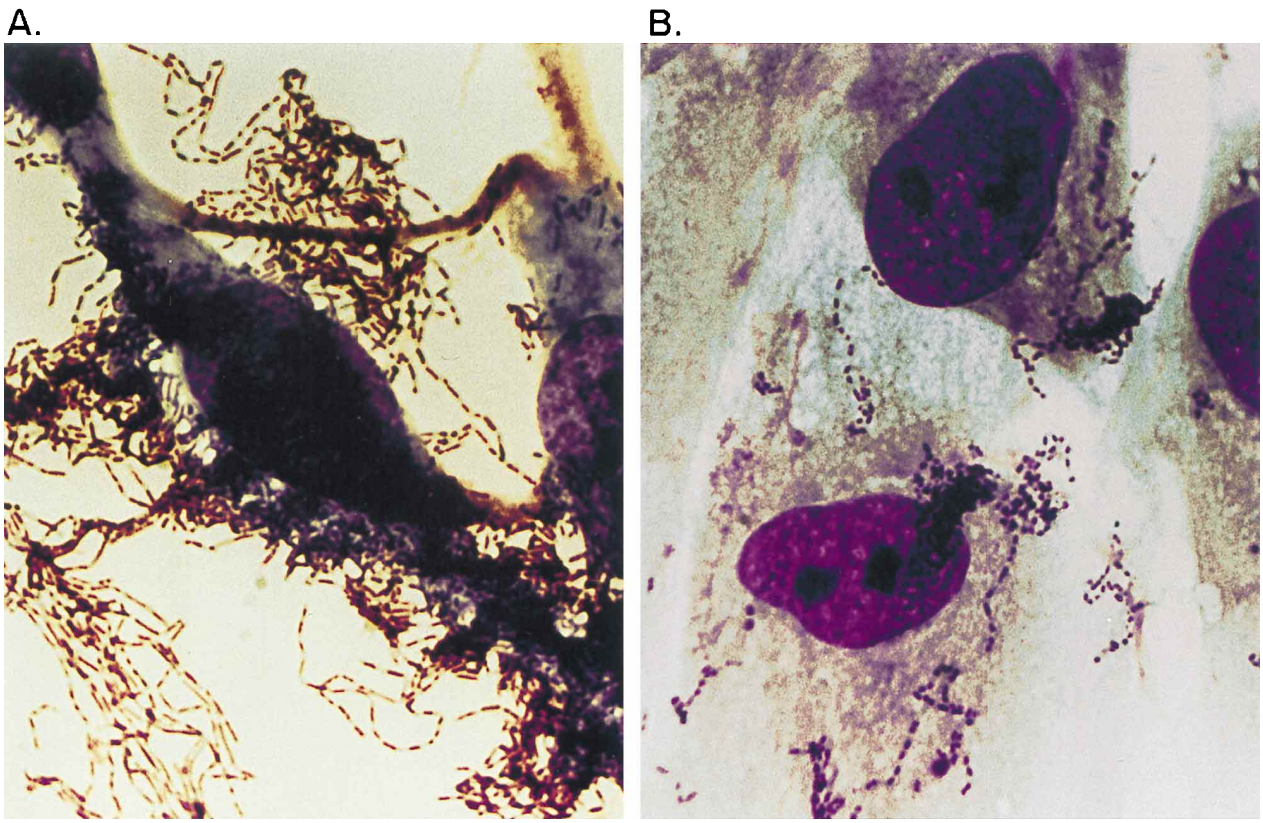

FIG. 6. Effect of altered expression levels of DnaK and GroEL on adherence of *H. ducreyi* to HFF. *H. ducreyi* 35000-PL, containing pLS88 (A), and 35000-KJ, containing pHDk4 (B), were used to inoculate monolayers of HFF (approximately 4 bacteria/HFF). Following 4 h of adherence at 32°C in E-FBS, nonadherent bacteria were washed off, fresh E-FBS was added, and incubation was continued for 20 h at 32°C before the coverslips were washed and stained with Diff-Quik. Adherent cells were photographed with a Nikon Labphot microscope under oil immersion (original magnification, ×1,000).

damaged essential proteins needed to multiply in vivo in the presence of accumulating heat stress coupled with oxidative stress.

Interestingly, stationary-phase *H. ducreyi*, regardless of GroEL level, was more sensitive than log-phase cells to combined heat and oxidative stresses (Table 1). These results are very different from those of previous studies with *E. coli*, where starvation was found to induce protection against both heat and hydrogen peroxide challenges (24, 29). Additionally, *E. coli* DnaK was found to be required for starvation-induced thermotolerance and hydrogen peroxide resistance (29). When DnaK was overproduced in stationary-phase *H. ducreyi* 35000-KJ, this organism became more sensitive than the wild type to oxidative and thermal stress. These results suggest that DnaK is not capable of protecting stationary-phase cells from environmental stress in *H. ducreyi*, while GroEL, which was found to increase slightly upon entry into stationary phase (Fig. 3), might offer some protection.

In addition, the long, tangled chains of *H. ducreyi* adhering to HFF were not seen in strain 35000-KJ containing altered levels of DnaK and GroEL (Fig. 6B). These results suggest that the high basal levels of GroEL are necessary for the proper folding of protein(s) needed for *H. ducreyi* chaining and/or adherence to host cells. Interestingly, the avirulent strains of *H. ducreyi*, A76 and A77, which are incapable of forming long adherent chains in the cell culture model (Fig. 4B), also expressed lower levels of GroEL (Fig. 1 to 3), providing another link between GroEL levels and adherence and/or chaining.

In summary, the results of this study demonstrate that the high level of GroEL is involved, either directly or indirectly, in the survival of stressed *H. ducreyi* and further suggest that a virulence-related phenotype (adherence and/or chaining) is influenced by cellular levels of GroEL.

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REFERENCES

1. **Abeck, D., A. P. Johnson, R. C. Ballard, Y. Dangor, E. A. Fontaine, and D. Taylor-Robinson.** 1987. Effect of cultural conditions on the protein and lipopolysaccharide profiles of *Haemophilus ducreyi* analysed by SDS-PAGE. FEMS Microbiol. Lett. **48:**397–399.
2. **Blum, P., J. Ory, J. Bauernfeind, and J. Krska.** 1992. Physiological consequences of DnaK and DnaJ overproduction in *Escherichia coli*. J. Bacteriol. **174:**7436–7444.
3. **Brown, T. J., J. Jardine, and C. A. Ison.** 1993. Antibodies directed against *Haemophilus ducreyi* heat shock proteins. Microb. Pathog. **15:**131–139.
4. **Buchmeier, N. A., and F. Heffron.** 1990. Induction of *Salmonella* stress proteins upon infection of macrophages. Science **248:**730–732.
5. **Cowing, D. W., J. C. A. Bardwell, E. A. Craig, C. Woolford, R. W. Hendrix, and C. A. Gross.** 1985. Consensus sequence for *Escherichia coli* heat shock gene promoters. Proc. Natl. Acad. Sci. USA **82:**2679–2683.
6. **Dixon, L. G., W. L. Albritton, and P. J. Willson.** 1994. An analysis of the complete nucleotide sequence of the *Haemophilus ducreyi* broad-host-range

plasmid pLS88. Plasmid **32:**228–232.
7. **Dolan, K. M., and E. P. Greenberg.** 1992. Evidence that GroEL, not σ^{32}, is involved in transcriptional regulation of the *Vibrio fischeri* luminescence genes in *Escherichia coli*. J. Bacteriol. **174:**5132–5135.
8. **Dunn, B. E., R. M. Roop II, C.-C. Sung, S. A. Sharma, G. I. Perez-Perez, and M. J. Blaser.** 1992. Identification and purification of a cpn60 heat shock protein homolog from *Helicobacter pylori*. Infect. Immun. **60:**1946–1951.
9. **Ericsson, M., A. Tärnvik, K. Kuoppa, G. Sandström, and A. Sjöstedt.** 1994. Increased synthesis of DnaK, GroEL, and GroES homologs by *Francisella tularensis* LVS in response to heat and hydrogen peroxide. Infect. Immun. **62:**178–183.
10. **Everest, P., G. Frankel, J. Li, P. Lund, S. Chatfield, and G. Dougan.** 1995. Expression of LacZ from the *htrA*, *nirB* and *groE* promoters in *Salmonella* vaccine strain: influence of growth in mammalian cells. FEMS Microbiol. Lett. **126:**97–101.
11. **Farr, S. B., and T. Kogoma.** 1991. Oxidative stress responses in *Escherichia coli* and *Salmonella typhimurium*. Microbiol. Rev. **55:**561–585.
12. **Fayet, O., T. Ziegelhoffer, and C. Georgopoulos.** 1989. The *groES* and *groEL* heat shock gene products of *Escherichia coli* are essential for bacterial growth at all temperatures. J. Bacteriol. **171:**1379–1385.
13. **Fisher, R. F., and S. R. Long.** 1992. *Rhizobium*-plant signal exchange. Nature **357:**655–660.
14. **Frydman, J., and F.-U. Hartl.** 1994. Molecular chaperone functions of hsp70 and hsp60 in protein folding, p. 251–283. *In* R. I. Morimoto, A. Tissiéres, and C. Georgopoulos (ed.), The biology of heat shock proteins and molecular chaperones. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
15. **Georgopoulos, C., K. Liberek, M. Zylicz, and D. Ang.** 1994. Properties of the heat shock proteins of *Escherichia coli* and the autoregulation of the heat shock response, p. 209–249. *In* R. I. Morimoto, A. Tissiéres, and C. Georgopoulos (ed.). The biology of heat shock proteins and molecular chaperones. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
16. **Govezensky, D., T. Greener, G. Segal, and A. Zamir.** 1991. Involvement of GroEL in *nif* gene regulation and nitrogenase assembly. J. Bacteriol. **173:** 6339–6346.
17. **Hansen, E. J., J. L. Latimer, S. E. Thomas, M. E. Helminen, W. L. Albritton, and J. D. Radolf.** 1992. Use of electroporation to construct isogenic mutants of *Haemophilus ducreyi*. J. Bacteriol. **174:**5442–5449.
18. **Herendeen, S. L., R. A. VanBogelen, and F. C. Neidhardt.** 1979. Levels of major proteins of *Escherichia coli* during growth at different temperatures. J. Bacteriol. **139:**185–194.
19. **Hoefer Scientific Instruments.** 1994. Hoefer protein electrophoresis applications guide. Hoefer Scientific Instruments, San Francisco, Calif.
20. **Jenkins, D. E., J. E. Schultz, and A. Matin.** 1988. Starvation-induced cross protection against heat or H_2O_2 challenge in *Escherichia coli*. J. Bacteriol. **170:**3910–3914.
21. **Kanemori, M., H. Mori, and T. Yura.** 1994. Effects of reduced levels of GroE chaperones on protein metabolism: enhanced synthesis of heat shock proteins during steady-state growth of *Escherichia coli*. J. Bacteriol. **176:**4235–4242.
22. **Kogoma, T., and T. Yura.** 1992. Sensitization of *Escherichia coli* cells to oxidative stress by deletion of the *rpoH* gene, which encodes the heat shock sigma factor. J. Bacteriol. **174:**630–632.
23. **Liberek, K., T. P. Galitski, M. Zylicz, and C. Georgopoulos.** 1992. The DnaK chaperone modulates the heat shock response of *Escherichia coli* by binding to the *sigma*^{32} transcription factor. Proc. Natl. Acad. Sci. USA **89:**3516–3520.
24. **Ma, M., and J. W. Eaton.** 1992. Multicellular oxidant defense in unicellular organisms. Proc. Natl. Acad. Sci. USA **89:**7924–7928.
25. **McCarty, J. S., and G. C. Walker.** 1994. DnaK mutants defective in ATPase activity are defective in negative regulation of the heat shock response: expression of mutant DnaK proteins results in filamentation. J. Bacteriol. **176:**764–780.
26. **Neidhardt, F. C., and R. A. VanBogelen.** 1987. Heat shock response, p. 1334–1345. *In* F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*; cellular and molecular biology. American Society for Microbiology, Washington, D.C.
27. **Parsons, L. M.** 1991. Differentiation of virulent and avirulent strains of *Haemophilus ducreyi* in a cell culture model, abstr. D-199. *In* Abstracts of the 91st General Meeting of the American Society for Microbiology. 1991. American Society for Microbiology, Washington, D.C.
28. **Parsons, L. M., A. L. Waring, and M. Shayegani.** 1992. Molecular analysis of *Haemophilus ducreyi groE* heat shock operon. Infect. Immun. **60:**4111–4118.
29. **Rockabrand, D., T. Arthur, G. Korinek, K. Livers, and P. Blum.** 1995. An essential role for the *Escherichia coli* DnaK protein in starvation-induced thermotolerance, H_2O_2 resistance, and reductive division. J. Bacteriol. **177:** 3695–3703.
30. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
31. **Scorpio, A., P. Johnson, A. Laquerre, and D. R. Nelson.** 1994. Subcellular localization and chaperone activities of *Borrelia burgdorferi* Hsp60 and Hsp70. J. Bacteriol. **176:**6449–6456.
32. **Shi, W., Y. Zhou, J. Wild, J. Adler, and C. A. Gross.** 1992. DnaK, DnaJ, and GrpE are required for flagellum synthesis in *Escherichia coli*. J. Bacteriol. **174:**6256–6263.
33. **Siegele, D. A., and R. Kolter.** 1992. Life after log. J. Bacteriol. **174:**345–348.
34. **Tilly, K., N. McKittrick, M. Zylicz, and C. Georgopolous.** 1983. The *dnaK* protein modulates the heat-shock response of *Escherichia coli*. Cell **34:**641–646.
35. **Totten, P. A., and W. E. Stamm.** 1994. Clear broth and plate media for culture of *Haemophilus ducreyi*. J. Clin. Microbiol. **32:**2019–2023.
36. **Trees, D. L., and S. A. Morse.** 1995. Chancroid and *Haemophilus ducreyi*: an update. Clin. Microbiol. Rev. **8:**357–375.
37. **Van Dyk, T. K., A. A. Gatenby, and R. A. LaRossa.** 1989. Demonstration by genetic suppression of interaction of GroE products with many proteins. Nature **342:**451–453.
38. **Yura, T., H. Nagai, and H. Mori.** 1993. Regulation of the heat-shock response in bacteria. Annu. Rev. Microbiol. **47:**321–350.
39. **Zeilstra-Ryalls, J., O. Fayet, L. Baird, and C. Georgopoulos.** 1993. Sequence analysis and phenotypic characterization of *groEL* mutations that block lambda and T4 bacteriophage growth. J. Bacteriol. **175:**1134–1143.
40. **Zhou, Y.-N., N. Kusukawa, J. W. Erickson, C. A. Gross, and T. Yura.** 1988. Isolation and characterization of *Escherichia coli* mutants that lack the heat shock sigma factor σ^{32}. J. Bacteriol. **170:**3640–3649.

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